FORM-PTO-1390 (Rev. 12-29-99) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 012627-021

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): 09/830981

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE99/03506	3 November 1999	3 November 1998

TITLE OF INVENTION: POLYPEPTIDE MEDIATING CELL PERMEABILITY

APPLICANT(S) FOR DO/EO/US: Eberhard HILDT; Stephanie SCHMIDT

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

JC08 Rec'd PCT/PTO 03 MAY 2001

U.S. APPLICATION NO. (If known,/ see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/830981	PCT/DE99/03506	012627-021

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00 (960)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00 (970)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00 (958)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00 (956)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00 (154)** for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	11 -20 =	0	X$18.00 (966)	$ --	
Independent Claims	1 -3 =	0	X$80.00 (964)	$ --	
Multiple dependent claim(s) (if applicable)			+$270.00 (968)	$	
TOTAL OF ABOVE CALCULATIONS =				$ 860.00	
Reduction for 1/2 for filing by small entity, if applicable (see below).				$ 430.00	-
SUBTOTAL =				$ 430.00	
Processing fee of **$130.00 (156)** for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 430.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 (581)** per property +				$	
TOTAL FEES ENCLOSED =				$ 430.00	
				Amount to be: refunded	$
				charged	$

a. ☒ Small entity status is hereby claimed.

b. ☒ A check in the amount of $ 430.00 to cover the above fees is enclosed.

c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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30,427
REGISTRATION NUMBER

Patent

Attorney's Docket No. 012627-021

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Eberhard HILDT et al	)	Group Art Unit: Unassigned
	)	
Application No.: Unassigned	)	Examiner: Unassigned
(Corresponds to PCT/DE99/03506)	)	
	)	
International Filing Date: 3 November 1999	)	
	)	
For: POLYPEPTIDE MEDIATING CELL PERMEABILITY	)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-captioned application as follows:

IN THE CLAIMS:

Kindly replace claims 1-3, 6 and 9-11 as follows:

1. (Amended) Polypeptide mediating cell permeability (CPP) , wherein CPP comprises the following amino acid sequence:

X o i i o X X i o X i X

in which

X = variable amino acid (hydrophilic, hydrophobic or with charged side groups)

o = hydrophobic amino acid

i = hydrophilic amino acid

wherein CPP is not a native HBV surface protein.

2. (Amended) The polypeptide mediating cell permeability (CPP) according to claim 1, wherein CPP comprises the amino acid sequence of figure 1 or an amino acid sequence differing therefrom by one or more amino acids and is not a native HBV surface protein and wherein the DNA sequence of the latter amino acid sequence hybridizes with the DNA of figure 1.

3. (Amended) A nucleic acid, coding for CPP according to claim 1.

6. (Amended) An expression plasmid, comprising the nucleic acid according to claim 3.

9. (Amended) An antibody directed against CPP according to claim 1.

10. (Amended) A method for mediating cell permeability to substances comprising using the CCP according to claim 1.

11. (Amended) The method according to claim 10, wherein the substances comprise polypeptides, nucleic acids and chemical compounds.

REMARKS

Entry of the foregoing amendments is respectfully requested.

Should the Examiner have any questions concerning the subject application, a telephone call to the undersigned would be appreciated.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: _____
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Date: May 3, 2001

Attachment to Preliminary Amendment dated May 3, 2001

Marked-up Claims 1-3, 6 and 9-11

1. (Amended) Polypeptide mediating cell permeability (CPP) , wherein CPP comprises the following amino acid sequence:

X o i i o X X i o X i X

in which

X = variable amino acid (hydrophilic, hydrophobic or with charged side groups)

o = hydrophobic amino acid

i = hydrophilic amino acid

wherein CPP is [no] not a native HBV surface protein.

2. (Amended) The polypeptide mediating cell permeability (CPP) according to claim 1, wherein CPP comprises the amino acid sequence of figure 1 or an amino acid sequence differing therefrom by one or more amino acids and is [no] not a native HBV surface protein and wherein the DNA sequence of the latter amino acid sequence hybridizes with the DNA of figure 1.

3. (Amended) A nucleic acid, coding for CPP according to claim 1 [or 2].

6. (Amended) An expression plasmid, comprising the nucleic acid according to claim 3[, 4 or 5].

Attachment to Preliminary Amendment dated May 3, 2001

Marked-up Claims 1-3, 6 and 9-11

9. (Amended) An antibody directed against CPP according to claim 1 [or 2].

10. (Amended) [Use of CPP according to claim 1 or 2] A method for mediating cell permeability to substances comprising using the CCP according to claim 1.

11. (Amended) [Use] The method according to claim 10, wherein the substances comprise polypeptides, nucleic acids and chemical compounds.

F 1704 6/PRTS

Polypeptide Mediating Cell Permeability

The present invention relates to a polypeptide which is cell-permeable and can mediate cell permeability to substances, to a DNA coding for such a polypeptide and a method of producing such a polypeptide. The invention also concerns antibodies directed against the polypeptide and the use of the polypeptide in the mediation of cell permeability to substances.

The property of substances penetrating cells is called cell permeability. This property is, however, only found in few substances. Most substances require auxiliary means and/or methods to penetrate cells. Examples thereof are microinjection, eletroporation, association with cationic lipids, liposome formation, receptor-mediated endocytosis and viral infection. However, these auxiliary means or methods involve great drawbacks. In particular, they are expensive, require complex experimental set-ups and can be used only to a limited extent. Their efficiency degree is also low and they are often toxic.

Therefore, it is the object of the present invention to provide a product by means of which substances can be inserted in cells, the above drawbacks being avoided.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on applicant's insights that a polypeptide comprising preferably the amino acid sequence

of figure 1 or an amino acid sequence differing therefrom by one or more amino acids may penetrate cells, i.e. has cell permeability. Applicant found such a polypeptide in the PreS2 region of a hepatitis B virus (HBV) surface protein. The polypeptide is referred to below as CPP "**c**ell **p**ermeability-mediating **p**olypeptide. CPP has the structure of an amphiphilic α-helix. Applicant also detected that one of CPP's properties is to mediate cell permeability to substances. The latter may then penetrate cells, the cell permeability of the substances not being limited to certain cells. The substances also retain their activities (cf. figure 2). Figure 3 shows variants of the CPP according to the invention from various HBV subtypes which differ from the sequence of figure 1 by one or more amino acids. The amino acid sequence of figure 1 (= PreS2-TLM) fully corresponds e.g. to subtype ayw (1) on an amino acid level, whereas the other subtypes have one or more replacements as compared thereto. However, it can be seen that conservation of certain amino acids exists between various HBV subtypes. It can be confirmed in particular by a graphic representation of hydrophobicity values. From this it is possible to draw the conclusion that even if one or more amino acids are replaced, the hydropathy distribution in the whole molecule should be retained. Due to this finding it is easily possible for a person skilled in the art to determine variants of the sequence of figure 1, since it is not the sequence as such that is decisive but the hydropathy profile in the whole molecule. The same applies correspondingly to various avian hepadnaviruses (cf. figure 4) or hepadnaviruses of rodents (cf. figure 5). Each of them is compared with a CPP according to figure 1 of the invention (= PreS2-TLM) and hydropathy profiles are prepared. They show that even with an almost complete replacement of the amino acids (e.g. HHBV <--> PreS2-TLM) the hydropathy

profile is substantially retained. This means that it is not the sequence as such that is decisive but the order of hydrophilic and hydrophobic amino acids in an α-helical motive. In a peptide comprising 12 amino acids, preferably positions 2, 5 and 9 are occupied by hydrophobic amino acids and positions 3, 4, 8, 11 are occupied by hydrophilic amino acids. The hydrophobic amino acids comprise valine, leucine, isoleucine, tryptophan, phenylalanine and methionine. The hydrophilic amino acids include glycine, serine, tyrosine, threonine, cysteine, asparagine and glutamine. A CPP according to the invention thus observes the following general formula:

X o i i o X X i o X i X

wherein X = variable amino acid (hydrophilic, hydrophobic or with charged side groups)
o = hydrophobic amino acid
i = hydrophilic amino acid

Amino acids having charged side groups are aspartate, glutamate (both have negatively charged side groups), lysine, asparagine, glutamine, arginine and histidine (all have positively charged side groups).

According to the invention applicant's insights are utilized to provide a polypeptide (CPP) which can mediate cell permeability to substances, CPP comprising the above indicated sequence or preferably the sequence of figure 1 or an amino acid sequence differing therefrom by one or more amino acids and being no native HBV surface protein and hybridizing the DNA of the latter amino acid sequence with the DNA of figure 1.

The term "mediating cell permeability to substances" refers to the fact that CPP can mediate cell permeability to substances of any kind and origin. The substances may be e.g. polypeptides (proteins), nucleic acids or chemical compounds. Examples of polypeptides are structural polypeptides, tumor necrosis factor, interferons, interleukins, lymphokines, growth factors, plasma proteins, e.g. blood clotting factors and metabolic enzymes, and receptors. In particular, the polypeptides may be those which may increase the immunogenicity of cells. These may be polypeptides not occurring in tumor cells, e.g. cytokines, such as IL-2, and GM-CSF, and co-stimulatory molecules, such as B7-1, tumor-associated antigens, e.g. MAGE1, tyrosinases and viral polypeptides, e.g. E7 of human papilloma virus and EBNA-3 polypeptide of Epstein-Barr virus. The polypeptides may also be adapter polypeptides, oligomerization motives of a polypeptide, polypeptide fragments of viral coat polypeptides, hormones and ribozymes. Examples of nucleic acids are those which may code for the above polypeptides. They may also be antisense oligonucleotides, peptide-nucleic acids and consensus sequences for transcription factors. Examples of chemical compounds are medicaments which have no polypeptide structure. These may be cytostatic agents, anesthetics, antihistaminics, antibiotics and antimycotics. For mediating cell permeability it may suffice to incubate CPP together with a substance so as to form chemical bonds, e.g. covalent or non-covalents bonds. It is favorable for CPP to be linked with the substance via a linker, which may be done e.g. via biotin/streptavidine. The linker may be available at the N-terminus or C-terminus of CPP. It is particularly advantageous for the substance to be present as polypeptide together with CPP in a fusion polypeptide. CPP may in this case be available at the N-terminus or C-terminus or within the polypeptide structure of the

substance. Therefore, the term "CPP" also comprises a fusion polypeptide in which CPP is present together with a substance. Mediation of cell permeability to substances may be detected by common methods. It is favorable to incubate cells with CPP-linked substances and detect the penetration or presence of CPP and/or the substances in the cells. This may be done e.g. by specific antibodies or reagents which react directly or indirectly with CPP and/or the substances.

The term "an amino acid sequence differing by one or more amino acids" comprises any CPP-preparing amino acid sequence which is not a native HBV surface protein. A sequence which codes for "an amino acid sequence differing by one or more amino acids" hybridizes preferably with the DNA sequence of figure 1. The DNA sequence may differ from the DNA of figure 1 by additions, deletions, substitutions and/or inversions of one or more base pairs. The term "hybridization" refers to a hybridization under common conditions, in particular at 20°C below the melting point of the sequence.

Another subject matter of the present invention is a nucleic acid which codes for CPP. The nucleic acid may be an RNA or a DNA. Preferred is a DNA which comprises the following:

(a) the DNA of figure 1 or a DNA differing therefrom by one or more base pairs, wherein the latter DNA hybridizes with the DNA of figure 1 and does not code for a natives HBV surface protein, or

(b) a DNA related to the DNA of (a) via the degenerated genetic code.

The expression "a DNA differing by one or more base pairs" comprises any DNA sequence coding for a CPP, which

hybridizes with the DNA of figure 1 and does not code for a native HBV surface protein. The DNA sequence may differ from the DNA of figure 1 by additions, deletions, substitutions and/or inversions of one or more base pairs. As to the expression "hybridization" reference is made accordingly to the above explanations.

A DNA according to the invention may be present as such or in a vector. In particular, a DNA according to the invention may be present in an expression vector. Examples thereof are known to the person skilled in the art. In the case of an expression vector for *E. coli* these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. For the expression in yeast e.g. pY100 and Ycpad1 have to be mentioned while e.g. pKCR, pEFBOS, cDM8, pCEV4, pCDNA3, pKSV10, pRCMV and pRK5 have to be indicated for the expression in animal cells. The bacculovirus expression vector pAcSGHisNT-A is particularly suitable for the expression in insect cells.

The person skilled in the art knows suitable cells to express the DNA according to the invention, present in an expression vector. Examples of such cells comprise the *E. coli* strains HB101, DH1, x1776, JM101, JM 109, BL21, SG 13009 and M15pRep4, the yeast strain *Saccharomyces cerevisiae*, the animal cells L, NIH 3T3, FM3A, CHO, COS, Vero, HeLa, Hep62, CCL13 and 293, the insect cells Sf9 and Sf21 and the plant cells *Lupinus albus*.

The person skilled in the art is familiar with methods and conditions of transforming or transfecting cells with an expression vector containing the DNA according to the invention and culturing the cells. He also knows methods of isolating and purifying the CPP expressed by the DNA according to the invention.

Another subject matter of the present invention is an antibody directed against CPP. Such an antibody may be prepared by common methods. It may be polyclonal or monoclonal. For its preparation it is favorable to immunize with CPP animals - in particular rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody. Further boosters of the animals may also be made with CPP. The polyclonal antibody may then be obtained from the animal serum or egg yolk. For preparing the monoclonal antibody, animal spleen cells are fused with myeloma cells.

Another subject matter of the present invention is a kit. Such a kit comprises one or more of the following components:

(a) a cell permeability-mediating polypeptide according to the invention (CPP),
(b) a DNA according to the invention,
(c) an antibody according to the invention, and
(d) common auxiliary agents, such as carriers, buffers, solvents, controls, etc.

One or more representatives of the individual components may be present each. As to the individual terms reference is made to the above explanations.

The present invention enables cell permeability to be mediated. Cell permeability can be mediated to substances of any kind and origin by a CPP according to the invention. The cell permeability is universal, i.e. it is not limited to certain cells. The cells may also be present *ex vivo* or *in vivo*. In addition, the cell permeability does not trigger any toxic effects.

The present invention is thus perfectly suited for diagnosis and therapy. The latter comprises influencing the expression of genes and metabolic processes. In particular, the present invention is suited for the diagnosis and therapy of the severest diseases, e.g. of tumors. The present invention distinguishes itself in particular in that it can be used for both conservative and gene-therapeutic measures.

Brief description of the drawings.

Figure 1 shows the amino acid and DNA sequences of a cell permeability-mediating polypeptide according to the invention (CPP).

Figure 2 shows the detection of CPP-mediated cell permeability. Lanes 2 and 3 show the activation of c-Raf1 kinase. Lanes 4 and 5 show the inhibition thereof. Lanes 6 and 7 show that mutated CPP-PLAP (CPP-KLAP) has no inhibitory effect.

Figure 3 shows the conservation of the amino acid sequence between various HBV subtypes as well as the hydropathy profile.

Figure 4 shows amphiphilic motives in the PreS region of various avian hepadnaviruses.

Figure 5 shows amphiphilic motives in the PreS2 region of various hepadnaviruses of rodents.

Figure 6 shows that DHBV42-53-EGFP is a cell-permeable protein.

The present invention is explained by the below examples.

Example 1: Detection of cell permeability mediated by a polypeptide (CPP) according to the invention.

The detection of cell permeability mediated by CPP is shown by inhibition of the TNFα-dependent activation of c-Raf1 kinase. The activation of the c-Raf1 kinase is based on the interaction between the TNF receptor I (TNR-RI) and the adapter molecule Grb2. For this purpose, the SH3 domain of Grb2 interacts with a PLAP motive from the cytoplasmic domain of TNF-RI.

CPP is provided in the form of a fusion polypeptide. In this fusion polypeptide referred to as CPP-PLAP CPP, the amino acid sequence of figure 1 is present as N-terminus partner and a PLAP motive from the cytoplasmic domain of TNF-RI is present as C-terminus partner. A fusion polypeptide referred to as CPP-KLAP is also provided, which has a mutated PLAP motive.

HeLa cells are incubated for 2 h with 2 µM CPP-PLAP or CPP-KLAP (control) and stimulated for 15 min with 100 µ/ml TNFα. The activation of c-Raf1 kinase is determined by an immunocomplex test using MEK (Santa Cruz, Biotech) and γ^{32}P-ATP as substrate (cf. figure 2).

It shows that CPP-PLAP reaches the cells and fully inhibits the activation of C-Raf1 kinase (cf. figure 2, lanes 4 and 5). It also turns out that CPP-KLAP does not achieve inhibition (cf. figure 2, lanes 6 and 7).

Example 2: Preparation and purification of a cell permeability-mediating polypeptide (CPP) according to the invention.

The DNA of figure 1 is provided at the 5' end with a BgIII linker and at the 3' end with a BamHI linker and is subsequently cleaved with the corresponding restriction enzymes. The resulting BgIII/BamHI fragment is inserted in the BamHI-cleaved expression vector pQe8 so as to obtain the expression plasmid pQe8/CPP.

One sequence coding for GST (glutathione S transferase) is also isolated from the expression plasmid pGex-1. At its 5' end it has a BamHI restriction site followed by a sequence coding for a thrombin restriction site. At its 3' end the sequence has a BamHI restriction site. The sequence is inserted in the BamHI-cleaved expression plasmid pQe8/CPP so as to obtain the expression plasmid pQe8/CPP-GST. It codes for the fusion polypeptide CPP-GST, pQe48/CPP-GST is used for the transformation of *E. coli* SG 13009 (cf. Gottesmann, S. et al., J. Bacteriol. 148 (1981), 265-273). The bacteria are cultured in an LB broth with 100 µg/ml ampicillin and 25 µg/ml kanamycin and induced for 4 h with 60 µM isopropyl-ß-D-thiogalactopyranoside (IPTG). Following induction, lysis of the sedimented and washed bacteria is carried out by means of ultrasound. The CPP-GST fusion polypeptide is isolated by means of affinity chromatography on a glutathione column. The bound CPP-GST fusion polypeptide is eluted from 0 to 10 mM by means of a linear increase in the glutathione concentration. The eluted CPP-GST fusion protein is subjected to thrombin cleavage. The hexa-His-CPP (fusion polypeptide) released in this way is subsequently isolated by affinity chromatography using denaturing conditions by means of an Ni-NTA agarose. This is done in the presence of

6 M urea in accordance with the instructions from the manufacturer (Quiagen company). The bound hexa-His-CPP is eluted in a buffer having pH 6.3, containing 250 mM imidazole. The hexa-His-CPP is subjected to 18 % SDS polyacrylamide gel electrophoresis and stained using coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J. Mol. Biol. 149 (1975), 709-733).

It shows that a (fusion) polypeptide according to the invention can be prepared in highly pure form.

Example 3: Preparation and detection of an antibody according to the invention

A fusion polypeptide of Example 2 according to the invention is subjected to 18 % SDS polyacrylamide gel electrophoresis. After staining the gel with 4 M sodium acetate, an about 3 kD band is excised out of the gel and incubated in phosphate-buffered common salt solution. Gel pieces are sedimented before the protein concentration of the supernatant is determined by SDS polyacrylamide gel electrophoresis followed by coomassie blue staining. Animals are immunized as follows with the gel-purified fusion polypeptide.

Immunization protocol for polyclonal antibodies in rabbits

35 µg of gel-purified fusion polypeptide in 0.7 ml PBS and 0.7 ml of complete or incomplete Freund's adjuvant are used per immunization:

Day 0: 1st immunization (complete Freund's adjuvant)

Day 14: 2nd immunization (incomplete Freund's adjuvant; icFA)

Day 28: 3rd immunization (icFA)

Day 56: 4th immunization (icFA)

Day 80: bleeding to death.

The rabbit serum is tested in an immunoblot. For this purpose, a fusion polypeptide of Example 2 according to the invention is subjected to SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10 (1984), 203-209). The Western blot analysis was carried out as described in Bock, C.-T. *et al.*, Virus Genes 8, (1994), 215-229. For this purpose, the nitrocellulose filter is incubated with a first antibody at 37°C for one hour. This antibody is the rabbit serum (1:10000 in PBS). After several wash steps using PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is an alkaline phosphatase-coupled monoclonal goat anti-rabbit IgG antibody (Dianova company) (1:5000) in PBS. 30 minutes of incubation at 37°C are followed by several wash steps using PBS and subsequently by the alkaline phosphatase detection reaction with developer solution (36 µM 5'-bromo-4-chloro-3-indolylphosphate, 400 µM nitro blue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature until bands are visible.

It shows that polyclonal antibodies according to the invention can be prepared.

Immunization protocol for polyclonal antibodies in chickens

40 µg of gel-purified fusion polypeptide in 0.8 ml PBS and 0.8 ml of complete or incomplete Freund's adjuvant are used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)

Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 50: 3rd immunization (icFA)

Antibodies are extracted from egg yolk and tested in a Western blot. Polyclonal antibodies according to the invention are detected.

Immunization protocol for monoclonal antibodies in mice

12 µg of gel-purified fusion polypeptide in 0.25 ml PBS and 0.25 ml of complete or incomplete Freund's adjuvant are used per immunization. In the fourth immunization, the fusion protein is dissolved in 0.5 ml (without adjuvant).

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 56: 3rd immunization (icFA)
Day 84: 4th immunization (PBS)
Day 87: fusion

Supernatants of hybridomas are tested in a Western blot. Monoclonal antibodies according to the invention are detected.

Example 4: Detection of the cell permeability mediated by DHBV-CPP

Detection of the cell permeability mediated by DHBV (duck hepatitis B virus)-CPP was made as follows: According to standard methods a fusion protein consisting of a hexa-His-Tag (6H), DHBV-CPP and eGFP (enhanced green fluorescent protein) was prepared in analogy to Example 1 in an *E. coli*

expression system. The pQE vector system of the Quiagen company was used. This protein (DHBV42-53eGFP) was isolated. wt6HeGFP (fusion protein of 6 His and eGFP) was used for control experiments. Then, 293 cells were incubated in the presence of these proteins for 10 and 20 minutes. The proteins were added to the medium at a concentration of 1 µM. After 10 or 20 minutes, the cells were lyzed and the cytosolic fraction of the cells was isolated by ultracentrifugation.

The presence of DHBV42-53-eGFP in the cytosol fraction was detected by means of Western blot analysis using a hexa-His-tag-specific antibody (figure 6, lanes 1-4) (anti-hexa-His6 of Qiagen company) or an eGFP-specific antibody (anti-eGFP of Clontech company) (figure 6, lanes 5-8). A peroxidase-conjugated secondary antibody (anti-mouse HRP, anti-rabbit-HRP of Amersham company) was used for the detection.

The Western blot shows that when DHBV42-53-eGFP is added an internalization of the protein into the cell (cytosol) can be observed after 10 min. (lanes 2, 6) or 20 minutes (4, 8) whereas in the case of the control protein wt6HeGFP which lacks the sequence mediating cell permeability this cannot be observed after either 10 minutes (lanes 1, 5) or 20 minutes (lanes 3, 7).

These results show that DHBV-CPP is capable of acting as a carrier for other proteins.

Claims

1. Polypeptide mediating cell permeability (CPP), wherein CPP comprises the following amino acid sequence:

X o i i o X X i o X i X

in which

X = variable amino acid (hydrophilic, hydrophobic or with charged side groups)
o = hydrophobic amino acid
i = hydrophilic amino acid

wherein CPP is no native HBV surface protein.

2. The polypeptide mediating cell permeability (CPP) according to claim 1, wherein CPP comprises the amino acid sequence of figure 1 or an amino acid sequence differing therefrom by one or more amino acids and is no native HBV surface protein and wherein the DNA sequence of the latter amino acid sequence hybridizes with the DNA of figure 1.

3. A nucleic acid, coding for CPP according to claim 1 or 2.

4. The nucleic acid according to claim 3, wherein the nucleic acid is a DNA.

5. A DNA according to claim 4, comprising:
   (a) the DNA of figure 1 or a DNA differing therefrom by one or more base pairs, wherein the latter DNA

hybridizes with the DNA of figure 1 and does not code for a native HBV surface protein, or

(b) a DNA related to the DNA of (a) via the degenerated genetic code.

6. An expression plasmid, comprising the nucleic acid according to claim 3, 4 or 5.

7. A transformant, containing the expression plasmid according to claim 6.

8. A method of preparing CPP, comprising culturing the transformant according to claim 7 under suitable conditions.

9. An antibody directed against CPP according to claim 1 or 2.

10. Use of CPP according to claim 1 or 2 for mediating cell permeability to substances.

11. Use according to claim 10, wherein the substances comprise polypeptides, nucleic acids and chemical compounds.

Abstract of the Disclosure

The present invention relates to a cell-permeable polypeptide that can mediate cell permeability to substances, DNA coding for said polypeptide and a method for the production of said polypeptide. The invention also relates to antibodies directed against the polypeptide and the use of said polypeptide in the mediation of cell permeability to substances.

P	L	S	S	I	F	S	R	I	G	D	P
CCC	ATA	TCG	TCA	ATC	TTC	TCG	AGG	ATT	GGG	GAC	CCT
	T										

Fig. 1

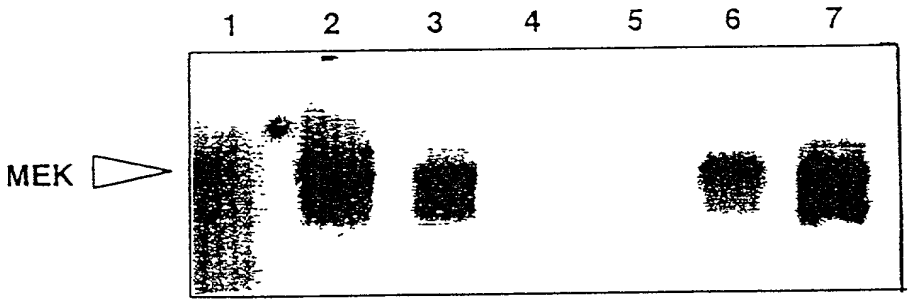


Fig. 2

Conservation of PreS2-TLM between various HVB subtypes

Nucleotide sequences, amino acid sequences and hydropathy values of the amino acid side chains (according to Kyte & Doolittle, 1982) of PreS2-TLM from subtype ayw (1) as compared to six other HBV subtypes ayw (2), adr (1), adr (2), ayr, adw and adw2. The amino acids identical with the sequence of PreS2-TLM of subtype ayw (1) and the associated hydropathy values are shown in boldface.

Subtype ayw (1)

CCC	TTA	TCG	TCA	ATC	TTC	TCG	AGG	ATT	GGG	GAC	CCT
Pro	**Leu**	**Ser**	**Ser**	**Ile**	**Phe**	**Ser**	**Arg**	**Ile**	**Gly**	**Asp**	**Pro**
-1.6	**3.8**	**-0.8**	**-0.8**	**4.5**	**2.8**	**-0.8**	**-4.5**	**4.5**	**-0.4**	**-3.5**	**-1.6**

Subtype ayw (2)

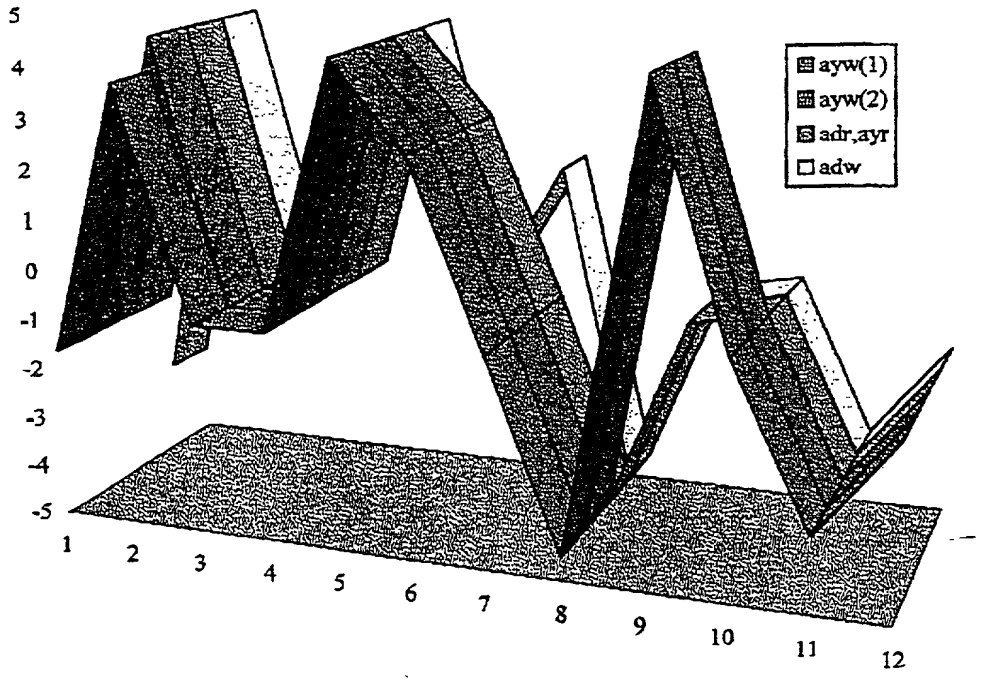
CCC	ATA	TCG	TCA	ATC	TTC	TCG	AGG	ATT	GGG	GAC	CCT
Pro	Ile	**Ser**	**Ser**	**Ile**	**Phe**	**Ser**	**Arg**	**Ile**	**Gly**	**Asp**	**Pro**
-1.6	4.5	**-0.8**	**-0.8**	**4.5**	**2.8**	**-0.8**	**-4.5**	**4.5**	**-0.4**	**-3.5**	**-1.6**

Subtypes adr (1), adr (2), ayr

CCC	ATA	TCG	TCA	ATC	TTC	TCG	AGG	ACT	GGG	GAC	CCT
Pro	Ile	**Ser**	**Ser**	**Ile**	**Phe**	**Ser**	**Arg**	Thr	**Gly**	**Asp**	**Pro**
-1.6	4.5	**-0.8**	**-0.8**	**4.5**	**2.8**	**-0.8**	**-4.5**	-0.7	**-0.4**	**-3.5**	**-1.6**

Subtypes adw, adw2

CAC	ATC	TCG	TCA	ATC	TCC	GCG	AGG	ACT	GGG	GAC	CCT
His	Ile	**Ser**	**Ser**	**Ile**	Ser	Ala	**Arg**	Thr	**Gly**	**Asp**	**Pro**
-3.2	4.5	**-0.8**	**-0.8**	**4.5**	-0.8	1.8	**-4.5**	-0.7	**-0.4**	**-3.5**	**-1.6**

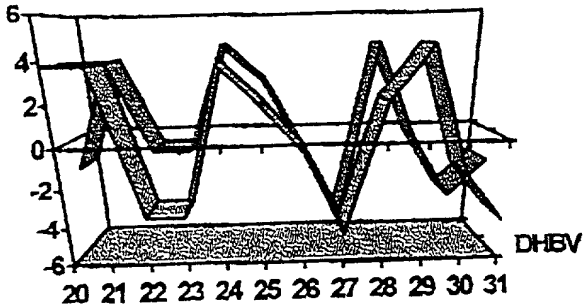


Amphiphilia of PreS2-TLM in various HBV subtypes

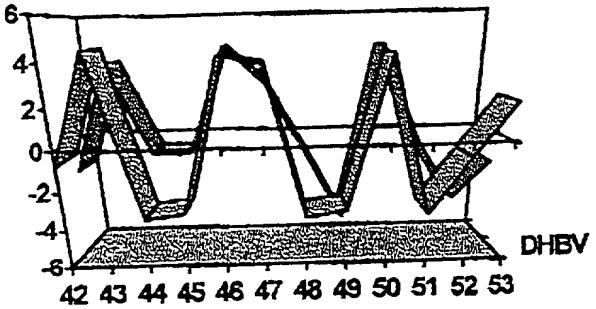
Representation of the distribution of the hydrophilic and hydrophobic amino acids in PreS2-TLM in subtype ayw (1) (blue) and the other HBV subtypes ayw (2) (green), adr (1), adr (2) ayr (red) as well as adw and adw2 (gray). The hydropathy values of the amino acid side chains (according to Kyte & Doolittle, 1982) are blotted on the y-axis, positive values correspond to hydrophobic amino acid side chains and negative values correspond to hydrophilic ones. The 12 amino acids of PreS2-TLM and the corresponding sequences of six other subtypes are plotted on the x-axis, the N-terminal prolin being located at position 1.

Fig. 3

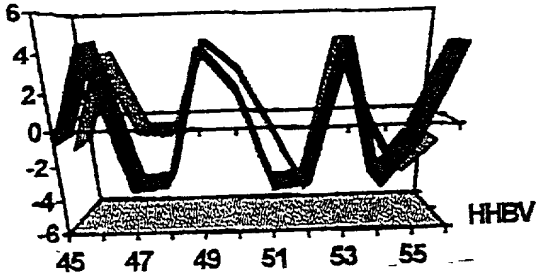
n	DHBV	PreS2-TLM	DHBV	PreS2-TLM
20	L (Leu)	P (Pro)	3,8	-1,6
21	L (Leu)	L (Leu)	3,8	3,8
22	N (Asn)	S (Ser)	-3,5	-0,8
23	Q (Gln)	S (Ser)	-3,5	-0,8
24	L (Leu)	I (Ile)	3,8	4,5
25	A (Ala)	F (Phe)	1,8	2,8
26	G (Gly)	S (Ser)	-0,4	-0,8
27	R (Arg)	R (Arg)	-4,5	-4,5
28	M (Met)	I (Ile)	1,9	4,5
29	I (Ile)	G (Gly)	4,5	-0,4
30	P (Pro)	D (Asp)	-1,6	-3,5
31	K (Lys)	P (Pro)	-3,9	-1,6



n	DHBV	PreS2-TLM	DHBV	PreS2-TLM
42	T (Thr)	P (Pro)	-0,7	-1,6
43	I (Ile)	L (Leu)	4,5	3,8
44	D (Asp)	S (Ser)	-3,5	-0,8
45	H (His)	S (Ser)	-3,2	-0,8
46	V (Val)	I (Ile)	4,2	4,5
47	L (Leu)	F (Phe)	3,8	2,8
48	D (Asp)	S (Ser)	-3,5	-0,8
49	H (His)	R (Arg)	-3,2	-4,5
50	V (Val)	I (Ile)	4,2	4,5
51	Q (Gln)	G (Gly)	-3,5	-0,4
52	T (Thr)	D (Asp)	-0,7	-3,5
53	M (Met)	P (Pro)	1,9	-1,6



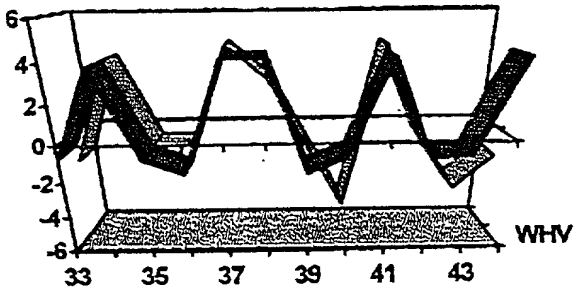
n	HHBV	PreS2-TLM	HHBV	PreS2-TLM
45	T (Thr)	P (Pro)	-0,7	-1,6
46	I (Ile)	L (Leu)	4,5	3,8
47	Q (Gln)	S (Ser)	-3,5	-0,8
48	H (His)	S (Ser)	-3,2	-0,8
49	V (Val)	I (Ile)	4,2	4,5
50	M (Met)	F (Phe)	1,9	2,8
51	D (Asp)	S (Ser)	-3,5	-0,8
52	H (His)	R (Arg)	-3,2	-4,5
53	I (Ile)	I (Ile)	4,5	4,5
54	D (Asp)	G (Gly)	-3,5	-0,4
55	S (Ser)	D (Asp)	-0,8	-3,5
56	V (Val)	P (Pro)	4,2	-1,6



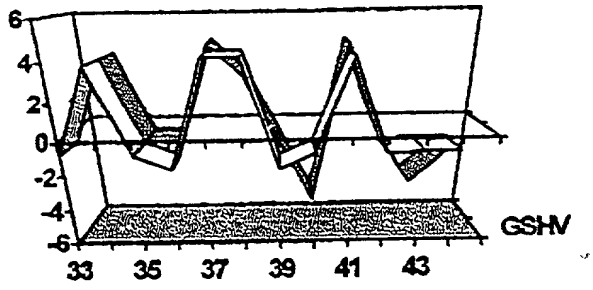
Amphiphilic motives in the PreS region of various avian hepadnaviruses
Comparison of the hydropathy profiles of PreS2-TLM (green) with segments of the PreS region of DHBV3 (red) and HHBV (blue). The positions of the amino acids in DHV3 and HHBV are indicated in the tables (n) together with the amino acid sequence of the corresponding segment and the PreS2-TLM. The hydropathy values are also indicated (according to Kyte & Doolittle, 1982). Motives having a distribution of hydrophobic and hydrophilic amino acids similar to that in PreS2-TLM are found between amino acids 20 to 31 aand 42 to 53 of DHBV3 or 45 to 56 of HHBV.

Fig. 4

n	WHV	PreS2-TLM	WHV	PreS2-TLM
33	T (Thr)	P (Pro)	-0,7	-1,6
34	L (Leu)	L (Leu)	3,8	3,8
35	S (Ser)	S (Ser)	-0,8	-0,8
36	P (Pro)	S (Ser)	-1,6	-0,8
37	V (Val)	I (Ile)	4,2	4,5
38	V (Val)	F (Phe)	4,2	2,8
39	P (Pro)	S (Ser)	-1,6	-0,8
40	T (Thr)	R (Arg)	-0,7	-4,5
41	V (Val)	I (Ile)	4,2	4,5
42	S (Ser)	G (Gly)	-0,8	-0,4
43	T (Thr)	D (Asp)	-0,7	-3,5
44	I (Ile)	P (Pro)	4,2	-1,6

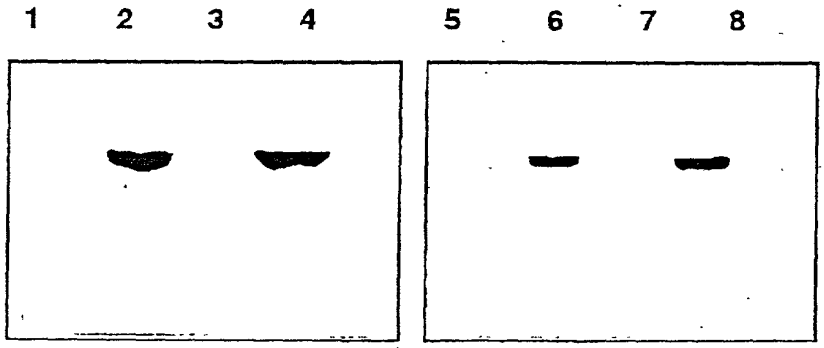


n	GSHV	PreS2-TLM	GSHV	PreS2-TLM
33	T (Thr)	P (Pro)	-0,7	-1,6
34	L (Leu)	L (Leu)	3,8	3,8
35	S (Ser)	S (Ser)	-0,8	-0,8
36	P (Pro)	S (Ser)	-1,6	-0,8
37	V (Val)	I (Ile)	4,2	4,5
38	V (Val)	F (Phe)	4,2	2,8
39	P (Pro)	S (Ser)	-1,6	-0,8
40	T (Thr)	R (Arg)	-0,7	-4,5
41	V (Val)	I (Ile)	4,2	4,5
42	S (Ser)	G (Gly)	-0,8	-0,4
43	T (Thr)	D (Asp)	-0,7	-3,5
44	T (Thr)	P (Pro)	-0,7	-1,6



Amphiphilic motives in the PreS2 region of various hepadnaviruses of rodents
A comparison of the hydropathy profiles of PreS2-TLM (green) with segments of the PreS region of WHV (turquois) and GSHV (yellow). The positions of the amino acids in WHV and GSHV are indicated in the tables (n) together with the amino acid sequence of the corresponding segment and the PreS2-TLM. The hydropathy values are also indicated (according to Kyte-& Doolittle, 1982). Motives having a distribution of hydrophobic and hydrophilic amino acids similar to that in PreS2-TLM are found between amino acids 33 to 44 of PreS2 from WHV and GSHV. The motive is conserved between both hepadnaviruses.

Fig. 5



DHBV42-53-EGFP is a cell-permeable protein
Immunoblot of cytosolic lyzates of 293 cells after 10 minutes (1, 2, 5, 6) or 20 minutes of incubation (3, 4, 7, 8) with 1 mM EGFP (1, 3, 5, 7) or 1 mM DHBV 42-53-EGFP (2, 4, 6, 8). For carrying out the immunoblot, an antibody directed against the N-terminal hexa-His-Tag of the recombinant proteins (1-4) or directed against EGFP was used (5-8).

Fig. 6

319-1US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY **(Includes Reference to Provisional and PCT International Applications)**	Attorney's Docket No. 012627-021

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name;
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:
POLYPEPTIDE MEDIATING CELL PERMEABILITY

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application
Number _____
on _____
and was amended
on _____ (if applicable).

☒ was filed as PCT international application
Number PCT/DE99/03506
on 3 November 1999
and was amended
on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(e) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

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COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. §119
DE	198 50 718.6	3 November 1998	X Yes _No
			_Yes _No
			_Yes _No
			_Yes _No
			_Yes _No

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(Application Number) (Filing Date)

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PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120:

U.S. APPLICATIONS		STATUS *(check one)*		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. APPLICATION NUMBERS ASSIGNED (if any)			

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF EIGHTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
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